

Award Number: W81XWH-08-1-0107

TITLE: Prolactin and Human Ovarian Cancer

PRINCIPAL INVESTIGATOR: Donyong Tan, Ph.D

CONTRACTING ORGANIZATION: The University of California Riverside
Riverside, CA 92521

REPORT DATE: September 2009

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

☒ Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188		
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-09-2009		2. REPORT TYPE Final		3. DATES COVERED (From - To) 15 Mar 2008 - 15 Aug 2009	
4. TITLE AND SUBTITLE Prolactin and Human Ovarian Cancer			5a. CONTRACT NUMBER		
			5b. GRANT NUMBER W81XWH-08-1-0107		
			5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Tan, Dunyong			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The University of California at Riverside Riverside, CA 92521			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Fort Detrick, Maryland 21702-5012			10. SPONSOR/MONITOR'S ACRONYM(S)		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Multiple prolactin receptors (PRLRs) derived from differential splicing of the same gene transcript transduce very different PRL-initiated effects on cells. In this project, we determined the kinds of PRLRs expressed by three human ovarian cancer cell lines: TOV-112D, OV-90 and TOV-21G. Using quantitative PCR and Western blot, we established that the long form (LF) and both short forms (SF1a and SF1b) of the human PRLRs were expressed by all three cell lines. To clarify the functional role of these receptors, we used three different ligands for the receptor, one which in other systems promotes proliferative (unmodified PRL, UPRL), one which acts as a competitive antagonist (G129R), and one, a selective activator (S179D). When cells were cultured in medium containing 10% FBS, UPRL had no effect on viable cell number while both of the other ligands reduced cell number. When FBS was omitted from medium, UPRL increased, while G129R and S179D decreased cell number. Further analysis showed UPRL decreased apoptosis and promoted cell migration, while S179D increased apoptosis and inhibited cell migration. The survival response and increased migration with UPRL and decrease in cell number and decreased migration in response to S179D were exaggerated by over expression of LF and SF1b.					
15. SUBJECT TERMS Prolactin, Prolactin receptor, ovarian cancer, apoptosis, survival					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 17	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

Page

Introduction.....	4
Body.....	5
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusion.....	7
References.....	8
Appendices.....	10

INTRODUCTION

Prolactin (PRL) is a pituitary polypeptide hormone characterized by multiple biological actions including proliferation and differentiation of mammary gland cells, the initiation and maintenance of lactation, immunoregulation, osmoregulation, behavior and reproduction (1-3). In the mammary gland during pregnancy, prolactin and the sex steroid hormones stimulate proliferation of the ductal epithelium into the surrounding mammary stroma, to produce lobulo-alveolar structures which are further stimulated by prolactin post-partum, resulting in lactation. Because of its mitogenic activity, in recent years, prolactin (especially extra-pituitary source) has emerged as a factor of relevance to a variety of cancers, especially reproductive cancers, such as breast cancer, prostate cancer (4,5).

The effects of PRL are mediated by interaction with specific receptors located on the plasma membrane of many target tissues (6, 7). PRL receptors (PRLR) belong to the superfamily of hematopoietic cytokine receptors, which are devoid of intrinsic catalytic activity (8). Rather, it associate with tyrosine kinases that are activated upon binding of the ligand, which in the case of the PRLR, results in one ligand-two receptor ternary complexes (8,9). There exists several prolactin receptor isoforms, including long form (LF), an intermediate form (IF) and two short forms (SF1a and SF1b) through mRNA splicing. Different isoforms mediate very different biological effects on the target cells after initiation of the cellular signal transduction by ligand binding. In the human, the best studied PRLRs to date include a long form (LF), a so-called intermediate form (IF) and two short forms (SF1a and SF1b). Each of these forms has the same extra-cellular sequence, but differs in the intra-cellular signaling region³. These variants of the PRLR have been shown to be the products of the same gene and arise by alternative splicing of the primary transcript. Work from several laboratories has suggested that the mitogenic and anti-apoptotic effects of PRL are dependent on the form(s) of PRLR expressed. In this regard, the LF has been associated in most studies with increased cell proliferation or anti-apoptotic effects while the SFs have been described as being dominant negatives by some groups and as producing cell cycle inhibitory signals and/or apoptotic signals by others. In addition, in the progression from healthy breast tissue to advanced breast cancer there is an increase in the LF:SF ratio (10).

Ovarian cancer remains the most lethal of the gynecological cancers. Although relatively uncommon, affecting about one of seventy women in the United States, the high mortality rate makes this disease a major health concern. Most ovarian cancer is derived from surface epithelial cells and these have been shown to express some form of the PRLR (11, 12), suggesting the potential of a growth response to PRL. In addition, peritoneal fluid contains PRL and the amount has been shown to reach levels of 8600 mIU/L in women with endometriosis, although no studies to date have examined patients with ovarian cancer. Thus, PRL is in significant concentrations in a compartment where it could contribute to the development or progression of ovarian cancer. Equally as important, is the possibility that the PRLR may serve as a specific marker of ovarian cancer cells within the peritoneum, allowing targeting of these cells during intra-peritoneal therapeutic treatment.

In the present study, by using regular and quantitative real time PCR as well Western blot, we determined the kinds of PRL receptor expressed by TOV-112D, OV-90 and TOV-21G cells. All three lines are epithelial and derived from malignant grade 3, stage III adenocarcinomas. The functional roles of prolactin and prolactin receptor in the growth, survival and migration of human ovarian cancer cells were also investigated.

BODY

Statement of Work Item (1)

Based on the experimental design of this award (Statement of work), the first important question we need to answer is whether or not prolactin receptor is expressed by human ovarian cancer cells and what kinds of prolactin receptor are expressed. Three human ovarian cancer cell lines including TOV21G, OV90 and TOV112D were cultured in medium (RPMI1640) containing 10% FBS in standard condition and total RNA was prepared using TRIzol reagent (Invitrogen). All three cell lines are epithelial and derived from malignant grade 3, stage III adenocarcinomas. After the first strand of complementary DNA was synthesized by using reverse transcriptase and oligo DNA primers, prolactin receptor mRNA (LF, SF1a and SF1b) were detected by using semi quantitative PCR and the primers specific to LF, SF1a and SF1b (see Appendix 1, Table 1). The amplicon size of LF, SF1a and SF1b are 989, 677 and 423 bps respectively by using these primers. As we expected, the PCR result showed that all three subtypes of prolactin receptor have been successfully detected in all three cell lines (Figure 1A).

The previous work in this lab and other lab evidenced that LF and two SFs (SF1a and SF1b) prolactin receptor play very different role in regulation of cell proliferation and differentiation as well as in characteristics of cancer cells. Work in DuFau's lab linked breast cancer to smaller ratio of SF:LF. To get the relative expression levels of three different subtypes of prolactin receptor, quantitative real time PCR (QPCR) was also applied and the same primers were used. As can be seen in Figure 1B and C (see appendix 1), in all three ovarian cancer cell lines, the LF is the dominant form of the receptor. In figure 1B and C, the relative mRNA of LF in TOV21G was considered as 1. The LF level in other two cell lines is a little bit above 1. Also, as can be seen in figure 1B, the relative expression level of SF1a and SF1b in TOV21G and OV90 are very similar (SF1a < SF1b), while in TOV112D is different where SF1a > SF1b. Whether or not this links to different characteristics of cancer cells needs further investigation.

Statement of Work Item (2)

To answer if the messenger RNA could be translated into protein, Western blot was also applied. The cells were treated in the same way as above, and the cell-lysate was prepared using lysis buffer containing protease and phosphatase inhibitor. The protein was detected by antibody against the extracellular domain of the receptor (PRLR-ECD) and the subtypes of the receptor were identified by their size (Fig 1 D). As shown in the figure, all three kinds of prolactin receptor have been successfully detected in their protein level.

Statement of Work Item (3)

The next question has to be answered is: whether or not these receptors functional? Have they any effect on human ovarian cancer cells? In order to solve this issue, the responsiveness of these cells to the UPRL, and its antagonists S179D and G129R was examined. Cells were seeded in 96 well plate (10^4 cells/well). After one-day recovery, cells were synchronized by incubation in serum-free RPMI 1640 for 24 hours. Then the cells were incubated in RPMI 1640 containing 10% FBS in the absence (Con) or presence of UPRL, S179D, G129R for a further 3 days. The viable cells were assayed by using MTS assay. As shown in Fig 2 A (see appendix 1), UPRL has no significant effect on the cell number of TOV21G, OV90 and TOV112D. While, both S179D and G129R decreased the cell number of TOV21G, OV90 and TOV112D in a dose-dependant manner (Fig 2).

Although UPRL has no significant effect on cell number when cells cultured in medium with FBS, the fact that its antagonists S179D and G129R decreases cell number imply a role of prolactin in the survival of these cells. To further address this issue, we examined the effect of UPRL and its antagonist S179 and G129R on the survival of cultured ovarian cancer cells. The results shows, compared to the control (serum free medium without any ligand), UPRL greatly

increases the survival of all three cell lines cultured in serum free medium in a dose dependant manner (Fig 3 A and B). However, its antagonists S179D and G129R have the opposite effect by decreasing the cell number (Fig 3 A).

Furthermore, we examined the effect of over-expression of LF or SF1b on the pro- and anti-survival effect of prolactin and S179D respectively. TOV112D cells were transiently transfected with expression plasmid containing LF or SF1b cDNA and the cells were cultured in serum free medium in the absence or presence of 500ng/mL of UPRL or S179D as indicated figure 3C. The result showed that over-expression of LF and SF1b significantly exaggerated the pro-survival effect of PRL and anti-survival effect of S179D (Fig 3C).

Although the above results shows that UPRL increases the cell number of all three ovarian cell lines cultured in medium without FBS (Fig 3), and S179D as well as G129R decreases the cell number of the cells cultured in medium with (Fig 2) or without FBS (Fig 3), the cell number could be the result of proliferation and apoptosis. To address this issue, we examined the effect of UPRL, S179D and overexpression of its receptor including LF and SF1b on the apoptosis rate of TOV112D by labeling the apoptotic DNA with fluorescein and scanned by confocal fluorescence microscope (Zeiss 510). The results shows that when the cells were cultured in serum free medium for three days, apoptosis rate (fluorecein labeled cells/PI stained cells) is about 8%. However, if in the presence of 500 ng/mL of UPRL or S179D, the rate is decreased to about 6% and increased to about 11% respectively. Also, the anti- and pro-apoptotic effect of UPRL and S179D were exaggerated by overexpressing long and short form receptor in these cells, the apoptosis rate for UPRL+LF and S179D+SF1b treated cells are 4% and 11.7% respectively (Fig 4).

To further clarify the possible mechanisms underlying the effect of UPRL and S179D on apoptosis of TOV112D cells, we also examined the effect of prolactin and S179D on expression of p21 and Akt by utilizing Western blot. As shown in Fig 4 B, S179D increases p21 protein expression in a dose dependant manner while UPRL has no significant effect on p21 expression. In regard of total Akt expression, both UPRL and S179D increases its expression.

Statement of Work Item (4)

The migration of TOV112D cells was examined by assessing the relative ability of the cells through 8 micron pores of a transwell culture insert system. Cells were 1) directly seeded in the upper chamber or 2) seeded in a 100mm plate, transfected with plasmids containing LF or SF1b cDNA and then splited into the upper chamber as indicated (see legend of Fig 6). The cells were cultured in medium containg 10% FBS and in the presence of PRL or S179D as indicated. After a 16 h migration period, cells on the original side of the membrane will be removed and those on the opposite side of the filter will be stained with crystal violet. Quantitation will be by photographic recording. As shown in Fig 6, prolactin significantly increases the migration of TOV112D cells and S179D has the ability of decreasing prolactin induced migration in a dose dependant fashion (Fig 6 A lower panel).

Also, by utilizing Western blot again, we examined the E-Cadherin protein. As shown in Fig 6C, we did not find any significant change of expression of E-Cadherin protein induced by S179D or S179D plus overexpression of SF1b. However, the E-Cadherin protein is down-regulated after administration of 500ng/mL of UPRL and UPRL plus LF overexpression.

Derivative experiments and results

The fact that G129R, which acts as a competitive antagonist to prolactin, reduced the cell number reminded us that there may exist a PRL autocrine system in ovarian cancer cells. In order to solidify this hypothesis, 1) the effect of antibody against human prolactin on the cell number was examined; 2) PRL mRNA was examined by RT-PCR using primers flanked the full coding sequence of human PRL. As can be seen in Fig 5, 1) PRL mRNA has been successfully detected in all the three ovarian cancer cell lines, 2) when cells were incubated in serum

supplemented, or serum free medium, in the presence of antibody against prolactin (1000µg/mL, 1:500), the cell number for all three cell lines are decreased (Fig 5 A).

KEY RESEARCH ACCOMPLISHMENTS

- Established that prolactin receptor including long form (LF) and two short form (SF1a and SF1b) are expressed in human ovarian cancer cells including TOV112D, OV90 and TOV21G (all three are epithelial cells, and malignant adenocarcinoma, grade 3, stage III).
- Established that unmodified PRL has no significant effect on, but its antagonists S179D PRL and G129R PRL, significantly decreases, the viable cell number of ovarian epithelial cancerous cells cultured in medium with 10% FBS.
- Established that unmodified PRL increases, S179D and G129R decreases the survival of ovarian cancer cells cultured in serum free medium.
- Using Apoptag fluorescein *in situ* apoptosis detection kit (Chemicon International, Inc.), Propidium Iodide (PI, 0.5-1.0 µg/mL) and confocal fluorescence microscopic technology, established the fact that Prolactin Inhibited, S179D promoted, apoptosis of cultured human ovarian cancer cells (TOV112D).
- Produced evidence suggesting that unmodified PRL promoted, S179D PRL and G129R PRL inhibited, cellular migration of TOV112D through through 8 micron pores of a transwell culture insert system.
- Produced evidence suggesting that some of the effect of unmodified PRL (such as promigration of TOV112D) and S179D PRL (such as decrease viable cell number) could be exaggerated by additional expression (transient transfection) of LF and SF1b respectively. This imply unmodified PRL and S179D may preferably use different receptors and different signal pathway system.
- Produced evidence suggesting involvement of PRL autocrine mechanism underlying growth and survival of ovarian cancer cells. Messenger RNA of prolactin was detected in human ovarian cancer cell lines suggesting these cells are potential sources of prolactin. Also, an inhibitory effect of antibody against prolactin on the growth of cultured ovarian cancer cells was observed..
- Evidenced that prolactin promotes, S179D and G129R inhibit, migration of cultured TOV112D.

REPORTABLE OUTCOMES

- Poster presentation “Evidence Supporting a Role for Prolactin in the Progression of Ovarian Cancer” at 100th AACR (Poster session abstract # 3887) held on February 3, 2009 at Denver, CO, USA.
- Manuscript for this study is in preparation and will be submitted soon.

CONCLUSION

Work on this award evidenced that prolactin receptor including long form (LF) and two short forms (SF1a and SF1b) are expressed at both mRNA and protein level in human ovarian cancer cell lines including TOV112D, OV90 and TOV21G.

UPLR has no effect on proliferation of ovarian cancer cells cultured in medium containing 10% FBS, however, this study evidenced that PRL is a strong survival factor for ovarian cancer cells when these cells cultured in FBS-free medium. The two antagonists (S179D and G129R) reduced viable cell number when the cells were cultured in FBS-containing or FBS-free medium.

The cell survival effect of PRL was increased with additional expression of LF receptor, whereas the effect of S179D on cell number was exaggerated with additional expression of SF1b receptor. The increased survival response to unmodified PRL and the decrease in viable cell number in response to S179D were exaggerated with additional expression (transient transfection) of LF and SF1b respectively.

UPRL has anti-apoptotic, while S179D has pro-apoptotic effect on cultured TOV112D cell lines.

UPRL significantly promoted, but S179D or G129R decreased, the cellular migration of TOV112D cells through 8 micron pores of a transwell culture insert system. The increased migration with UPRL and decreased migration in response to S179D PRL were exaggerated by overexpression (transient transfection) of LF and SF1b respectively.

PRL autocrine mechanism underlying cell growth and Survival is involved in ovarian cancer cells.

These results, while modest in the short term, suggest different roles for the LF and SF 1b receptor, an important role for unmodified prolactin in survival and migration of ovarian cancer cells, and potential therapeutic applications for S179D PRL and G129R PRL.

REFERENCES

1. U.S. Department of Health and Human Services. United States Cancer Statistics: 1999-2002 Incidence and mortality web-based report. www.cdc.gov/cancer/npcr/uscs/.
2. Christine Bole-Feysot, Vincent Goffin, Marc Edery, Nadine Binart, and Paul A. Kelly. Prolactin (PRL) and its receptor: Actions, Signal transduction pathways and Phenotypes Observed in PRL receptor knockout mice. *Endocrine Reviews* 1998; 19(3): 225–268.
3. Goffin V, Kelly PA 1997. The prolactin/growth hormone receptor family: structure/function relationships. *J Mammary Gland Biol Neoplasia* 2:7-17.
4. Charles V. Clevenger. Roles and Regulation of Stat Family Transcription Factors in Human Breast Cancer. *American Journal of Pathology*, 2004; 165(5)
5. Watson CJ: Stat transcription factors in mammary gland development and tumorigenesis. *J Mammary Gland Biol Neoplasia* 2001, 6:115– 127
6. JF Trott, RC Hovey, S Koduri, and BK Vonderhaar. Alternative splicing to exon 11 of human prolactin receptor gene results in multiple isoforms including a secreted prolactin-binding protein. *Journal of Molecular Endocrinology*. 2003, 30 (1): 31-47.
7. Ameae M. Walker S179D Prolactin Antagonistic Agony! *Mol Cell Endocrinol*. 2007 September 30; 276(1-2): 1–9.
8. Walker AM. Prolactin receptor antagonists. *Curr Opin Investig Drugs*. 2005;6(4):378-85.
9. Vincent Goffin*, Estelle Tallet, Jean-Baptiste Jomain and Paul A. Kelly. Development of Prolactin Receptor Antagonists: Same Goal, Different Ways. *Recent Patents on Endocrine, Metabolic & Immune Drug Discovery* 2007, 1: 41-52 41

10. Jianping Meng, Chon-Hwa Tsai-Morris and Maria L. Dufau. Human prolactin receptor variants in breast cancer. *Cancer Research* 2004; 64:5677-5682.

11. Hiromi Kinoshita, Toshiyuki Yasui, Kenjiro Ushigoe, Minoru Irahara, Minoru Tanaka, Kunio Nakashima, Toshihiro Aono. Expression of ovarian prolactin receptor in relation to hormonal changes during induction of ovulation in the rat. *Gynecologic and Obstetric Investigation*. 2001; 52:132-138.

12. Mikiko Asai-Sato, Yoji Nagashima, Etsuko Miyagi, Ken Sato, Ichiro Ohta, Barbara K. Vonderhaar, Fumiki Hirahara. Prolactin Inhibits Apoptosis of Ovarian Carcinoma Cells Induced by Serum Starvation or Cisplatin Treatment. *International J of Cancer*. 2005; 115(4): 539-544

APPENDICES

Appendix 1: Table 1 and Figure 1-6

Table 1 Primers used to detect prolactin receptors

	Sequences	Length of Amplicon
LF	S/A: 5' TAAATGGTCTCCACCTACCCTGAT 3' /5' CCTCCCTCTGCTGGGTTGCCTTT 3'	989 bp
SF1a	S/A: 5' TAAATGGTCTCCACCTACCCTGAT 3' / 5' CTGTGGTCAATGTTGCCTTTGTG 3'	677 bp
SF1b	S/A: 5' TAAATGGTCTCCACCTACCCTGAT 3' /5' CACCTCCAACAGATGAGCATCAAATCC 3'	412 bp

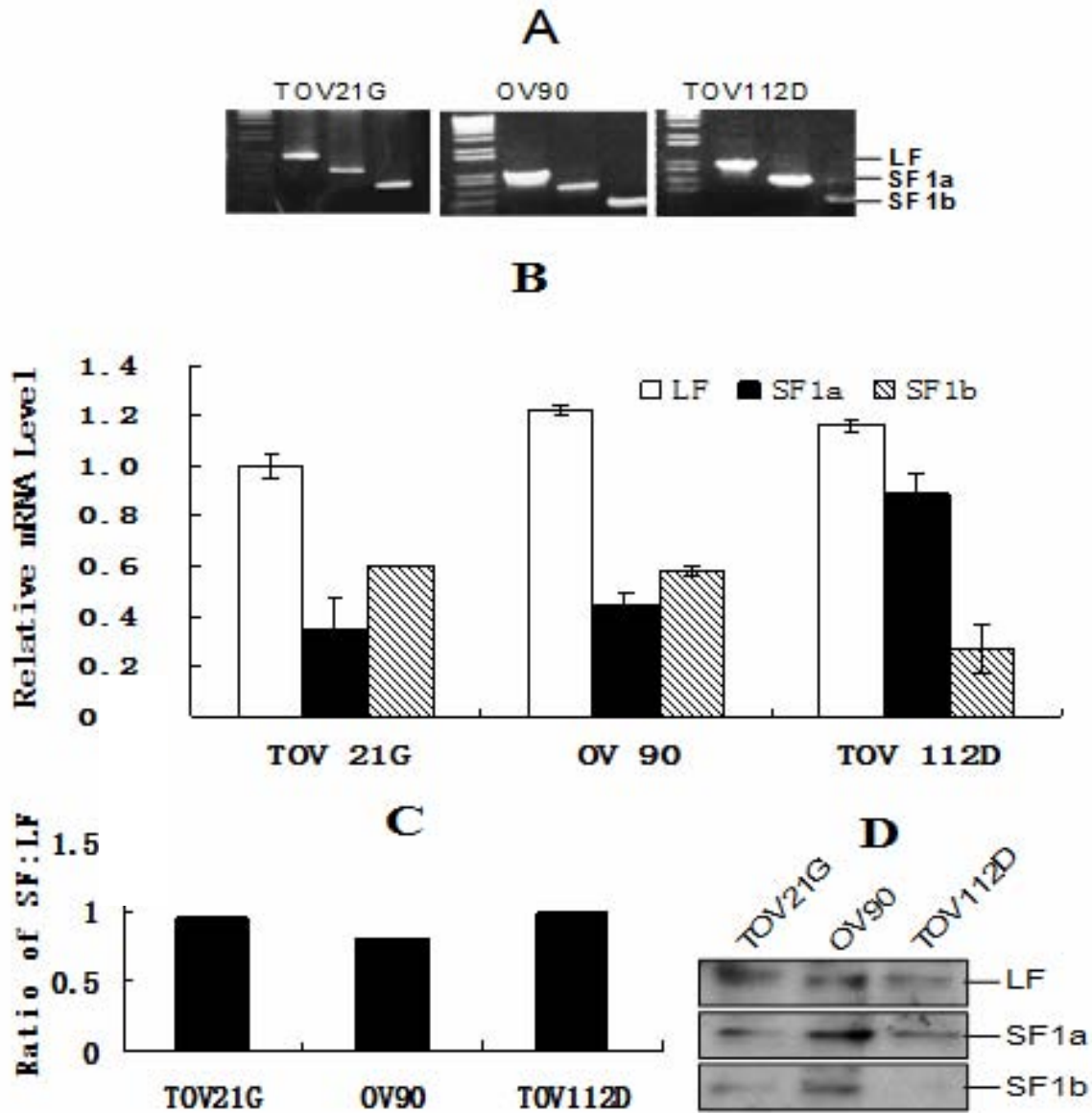


Fig 1 PRLR expression in human ovarian cancer cells. Ovarian cancer cells (TOV21G, OV90 and TOV112D) were cultured in RPMI 1640 medium containing 10% FBS one day prior to the total RNA extraction (A and B) using TRIzol reagent (Invitrogen) and preparation of cell lysate (C) using cell lysis buffer containing protease and phosphatase inhibitor as described in methods. First strand of cDNA was achieved by reverse transcription using reverse transcriptase M-MLV (Promega). The relative mRNA of LF, SF1a and SF1b was measured by semi-quantitative (A) and quantitative real time PCR and the primers used were shown in Table 1 (B). LF, SF1a and SF1b protein was determined by western blot using antibody against the extra-cellular domain of PRLR (PRLR-ECD), and LF, SF1a and SF1b proteins were identified by their size (C).

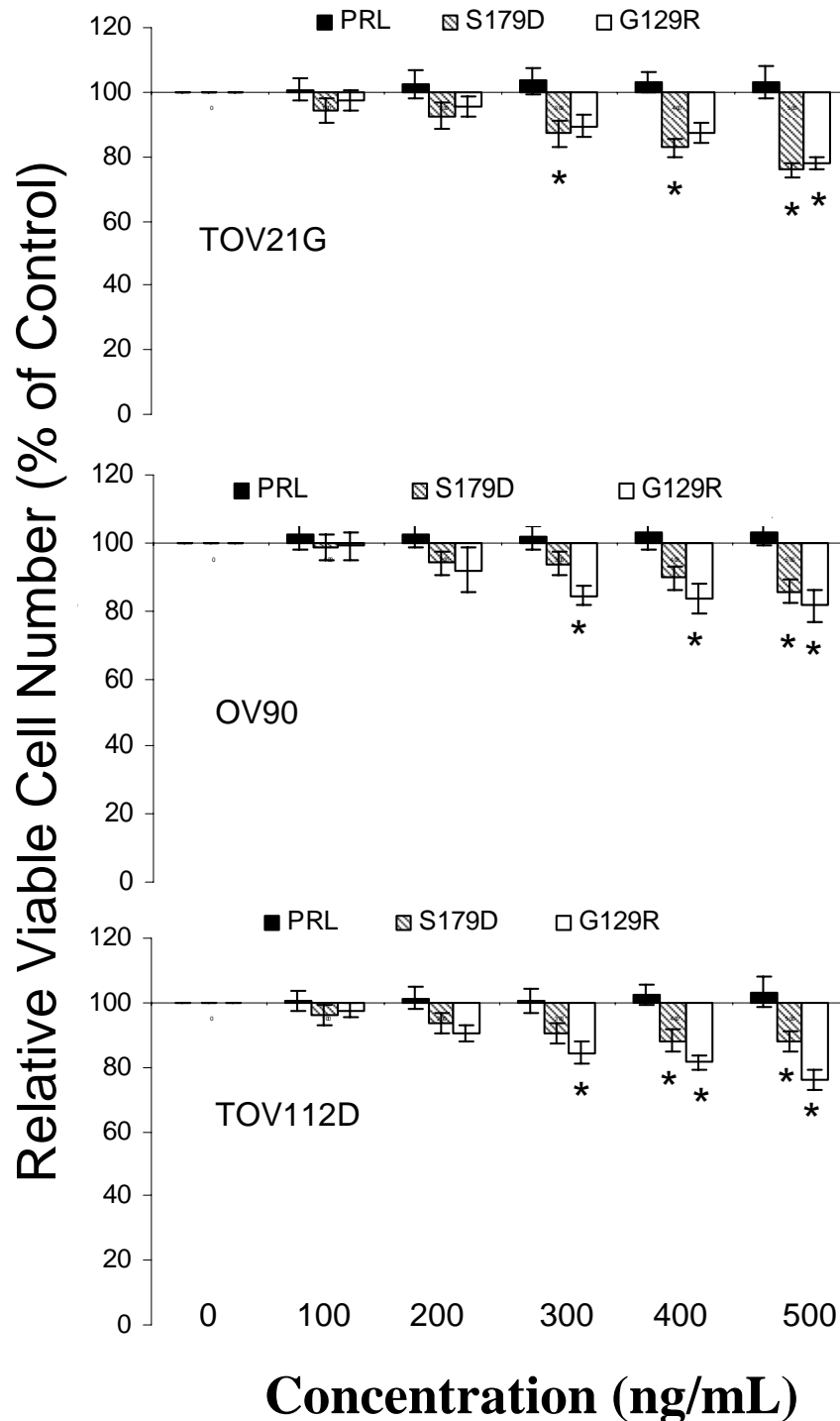


Fig 2 Effect of PRL and its antagonists, S179D and G129R on viable cell number of ovarian epithelial cancerous cells cultured in medium with 10% FBS Ovarian cancer cells (TOV21G, OV90 and TOV112D) were seeded in 96-well plate and cultured in RPMI 1640 medium containing 10% FBS in the absence (Con) or presence of 0-500ng/mL of PRL, S179D and G129R. After a further 72h-incubation, the relative viable cell number was determined by MTS assay (viable cell number is positively related to the conversion of MTS into a soluble formazan, which was measured by the absorbance of at 492 nm). * $p < 0.05$, ** $P < 0.01$ versus control (0 ng/mL of PRL, S179 or G129R).

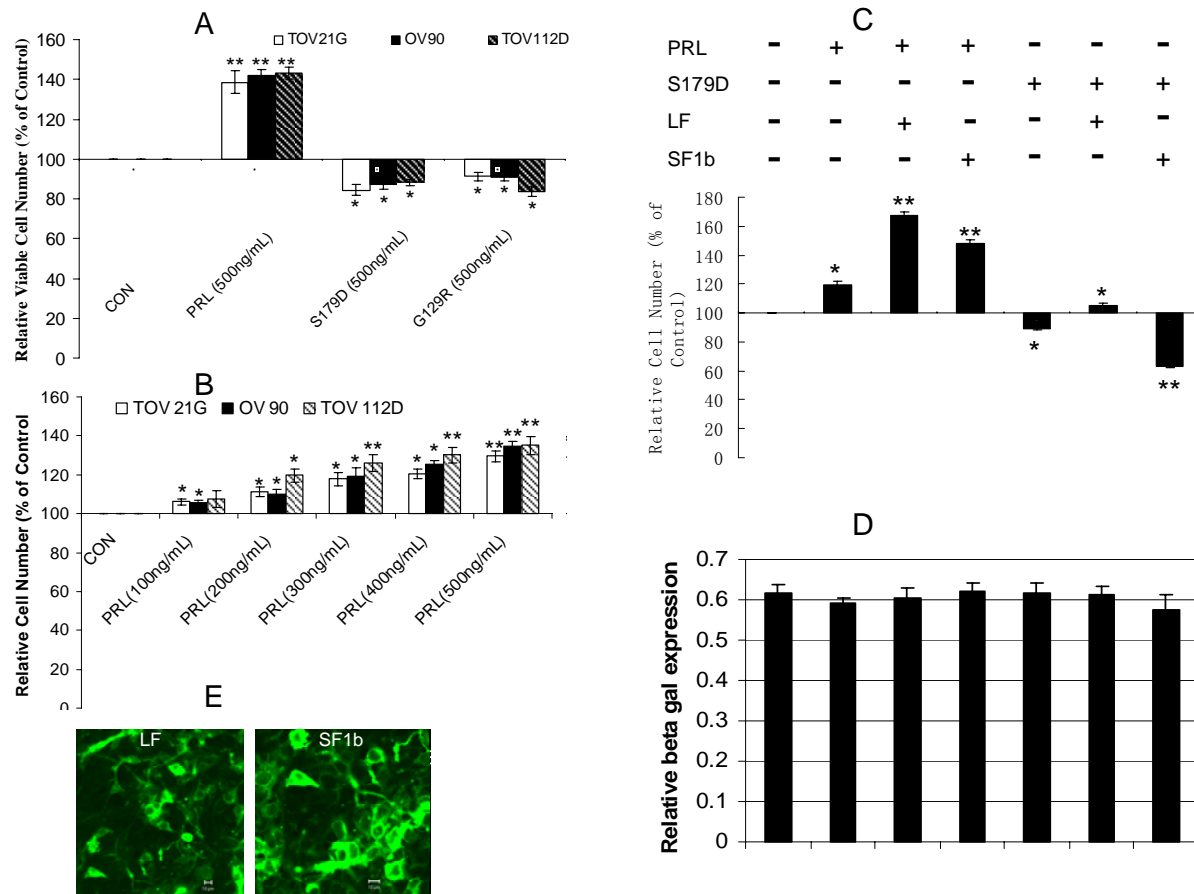


Fig 3 Effect of PRL, S179D and G129R on survival of ovarian cancer cells TOV21G, OV90 and TOV112D cells were seeded in 96-well plate. A. The cells cultured in serum free RPMI 1640 medium in the absence (Con) or presence of 500ng/mL of PRL, S179D and G129R. After 1 seven-day incubation (37°C, 4% CO₂), the relative viable cell number was determined by MTS assay as in Fig 2. B. the cells were cultured in the absence (Con) or presence of 100ng/mL – 500ng/mL of PRL and the MTS assay was carried out after seven-day-incubation. C. TOV112D cells were transiently transfected with or without pLF or pSF1b as indicated in the figure. After 24 h-recovery, the cells were incubated in medium without FBS and in the absence or presence of 500ng/mL of prolactin or S179D as indicated in the Fig for seven days (the equal amount of total DNA for each set of cells were achieved by irrelevant plasmid, pRedTandem, which express red protein). Viable cell number assay was conducted as above. The lower panel shows relative expression of β -gal to confirm the equal transfection efficiency. This was carried out in parallel with a separate set of cells 48 hours after transfection. D. A separate set of cells were transiently transfected with pLF-GFP or pSF1b-GFP and examined by confocal fluorescence microscope to confirm the transfection efficiency in TOV112D cells. *, P<0.05, **, P<0.01 versus control.

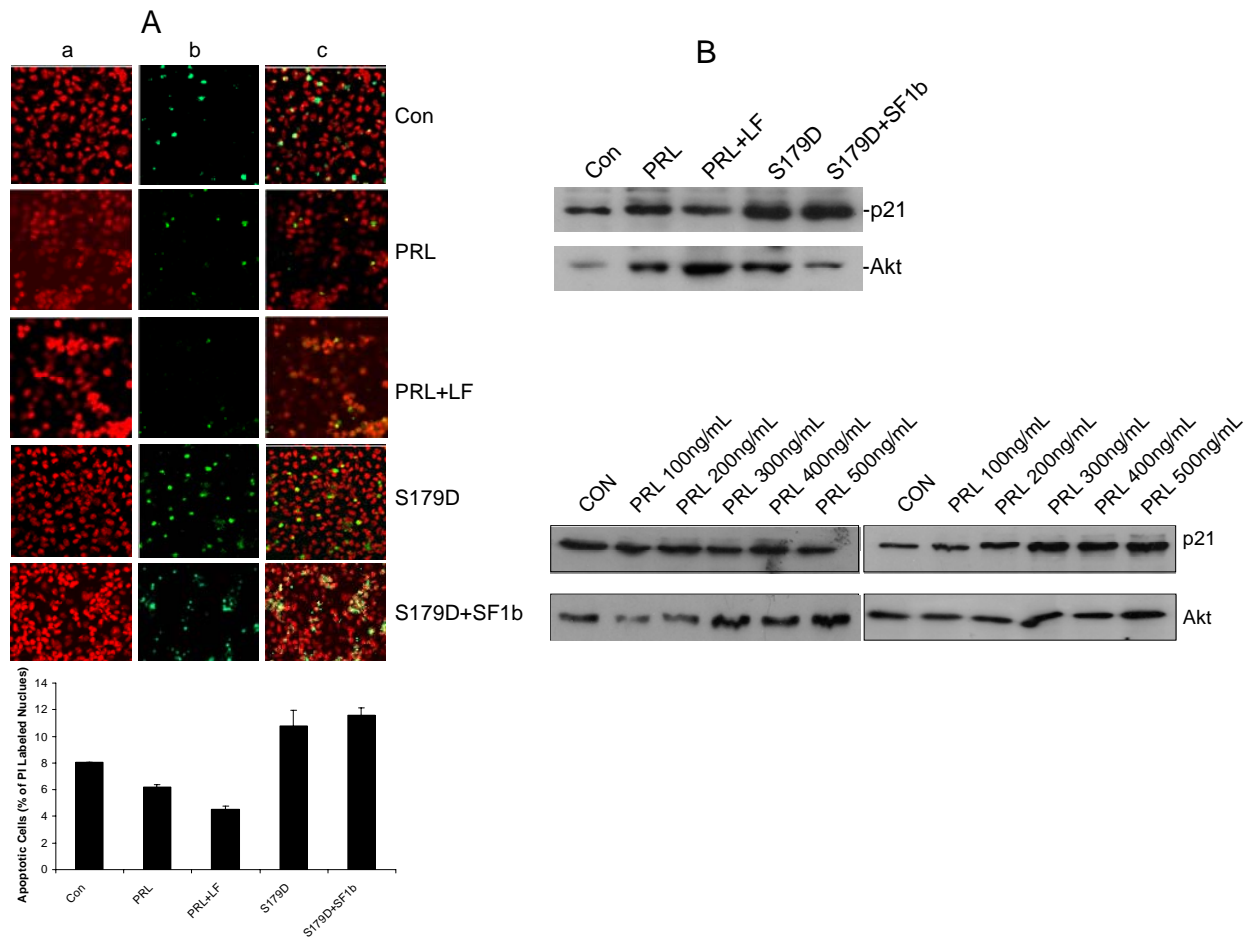


Fig 4 Effect of PRL and S179D on apoptosis of ovarian cancer cells A (upper panel): Confocal image shows apoptotic cells. TOV112D cells were seeded on coverslip ($\Phi=12\text{mm}$) preplaced in six-well plate (5×10^5 cells/well) one day prior to transfection. The next day, by using lipofectamine 2000 (Invitrogen, CA), the cells (80% confluency) were transfected with expression plasmid containing cDNA of LF or SF1b as indicated and the cells not designed to transfected with pLF or pSF1b were transfected with irrelevant plasmid, pLUC, which would express an irrelevant protein. After a 12 h recovery, the medium was changed to serum free medium and the cells were cultured in the absence (Con) or presence of 500ng/mL of PRL or S179D (as indicated in the Fig) for 72 h. Apoptotic cells were fluorescently labeled using Apoptag fluorescein *in situ* apoptosis detection kit (Chemicon International, Inc.) and Propidium Iodide (PI, 0.5-1.0 $\mu\text{g/mL}$) was used for counterstain. The apoptotic and total nucleus stained by fluorescein and PI respectively were examined and scanned by confocal fluorescence microscope (Zeiss 510, Germany) using multi-track and the filters of 488nm/505nm (for fluorescein) and 543nm/560nm (for rhodamine). a, PI stained nucleus; b, fluorescein labelled apoptotic nucleus; c, combination of a and b. A (lower panel): Histograms show the average percentage of apoptotic cells as a proportion of the total PI stained cells. * $p < 0.05$ vs control cells. B. Western blot shows effect of PRL and S179D on expression of p21 and Akt. Upper panel: TOV112D cells seeded in six-well plate were transiently transfected with pLF or pSF1b as indicated or irrelevant plasmid pRedTandem for rest of the cells. After 12h recovery, cells were incubated in serum free RPMI1640 and in the absence (Con) or presence of 100ng/mL – 500ng/mL of PRL or S179D for 24h. Lower panel: cells were incubated in the absence (Con) or presence of 100ng/mL – 500ng/mL of PRL or S179D. The cell lysate preparation and Western blot was carried out as described in method section.

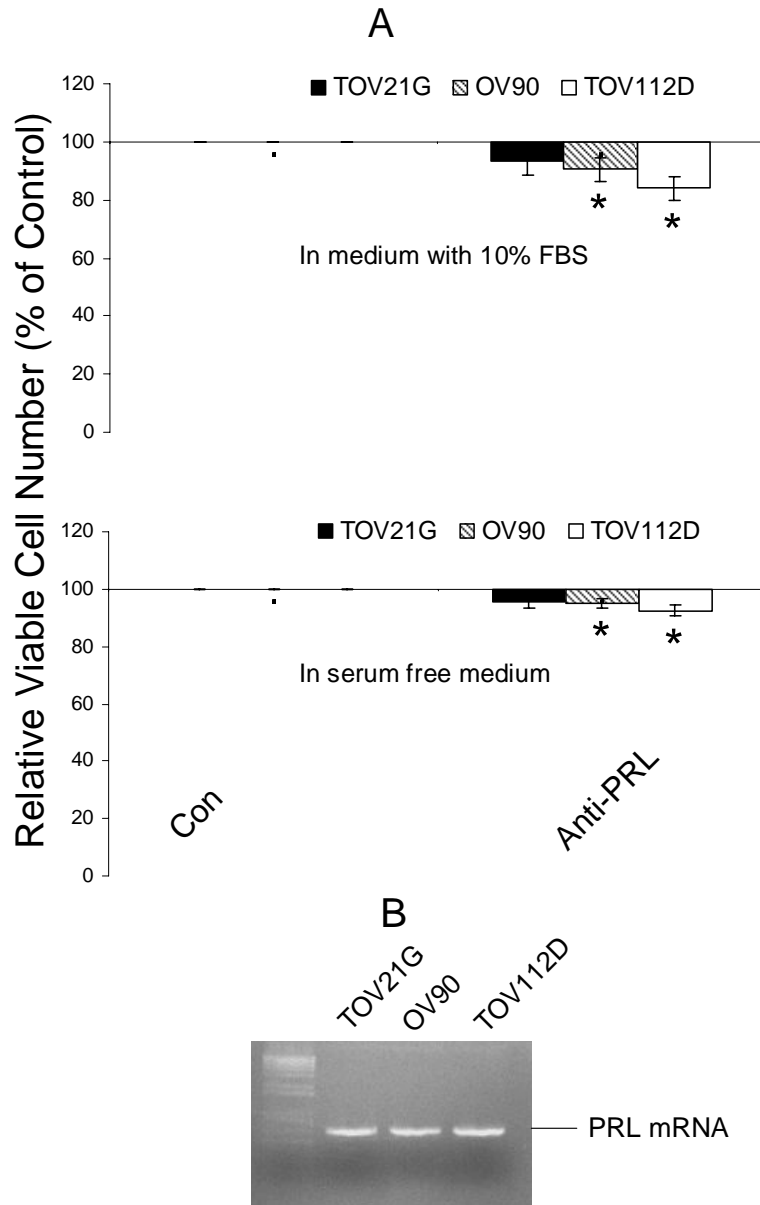


Fig 5 Involvement of PRL-Autocrine Mechanism in Survival of Ovarian Cancer Cells A. Human ovarian cancer cells (TOV21G, OV90 and TOV112D) were cultured in medium with (upper panel) or without (lower panel) 10% FBS in the absence (Con) or presence of antibody against human prolactin or 500ng/mL of G129R, or both as indicated. The cell number was determined by using MTS assay as above after 3 day (upper panel) or 6 day (lower panel) incubation. B. PRL mRNA in human ovarian cancer cells. TOV21G, OV90 and TOV112D cells were cultured in a standard condition. Total RNA extraction and cDNA preparation were conducted as in Fig 1. PRL mRNA was examined by regular PCR and the primers used are: 5'-ATG AAC ATC AAA GGA TCG-3'(forward)/5'- GCA GTT GTT GTT GTG GGA TGA-3'(reverse), which would amplify the full sequence coding PRL. C. PRL protein in human ovarian cancer cells. *, $P < 0.05$, **, $P < 0.01$ versus control.

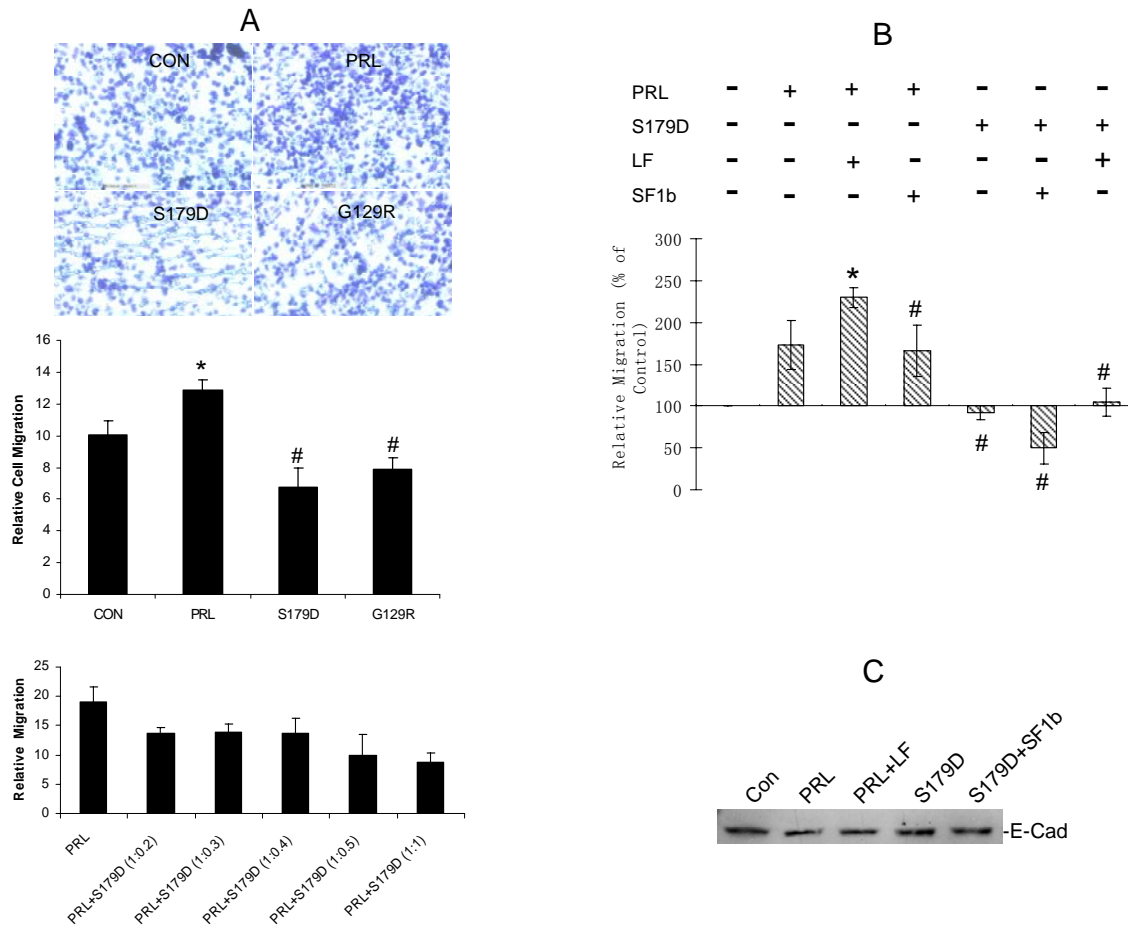


Fig 6 Effect of PRL, S179D on migration of TOV112D TOV112D cells were seeded in a transwell chamber (6.5mm in diameter and 8μm in pore size). A. Cells were cultured in RPMI 1640 medium containing 10% FBS in the absence (Con) or presence of 500ng/mL of PRL, S179D and G129R or 500mg/mL of PRL plus different dose of S179D (from 1:0 – 1:1 by the ratio of PRL: S179D). After 20-h incubation, the cells on the original side of the membrane were removed by steady scraping using cotton swab tipped applicators, and the cells on the other side of the filter were stained using crystal violet (1% tuluid blue in PBS). Finally the stained cells were recorded photographically and relative migrating cells were represented by the heaviness of the stain which was measured using Kodak Imager System. B. Cells were cultured in a 100 mm plate were transiently transfected with or without pLF or SF1b (as indicated) and after a 24h-recovery, cells were detached by trypsinization and seeded in the transwell chamber as above and incubated in the absence (Con) or presence of 500ng/mL of PRL or S179D as indicated. The migration were assayed (as in A) 20 h after addition of the ligands. C. Effect of PRL and S179D on expression of E-Cadherin in TOV112D cells. Cells were seeded in six-well plate one day prior to the transfection of pLF or pSF1b. After 12 h recovery, the cells were incubated in the absence or presence of 500ng/mL of PRL or S179D as indicated for a further 24 h. Cell lysate and Western blot were conducted as described in method. * $p < 0.05$, vs Con, # $p < 0.05$ vs PRL in A; * $p < 0.05$ vs PRL in B; * $p < 0.05$ vs the cells without transient over-expression of LF or SF1b and without any ligand administration.

Appendix 2: Abstract for AACR meeting

Evidence Supporting a Role for Prolactin in the Progression of Ovarian Cancer

Dunyong Tan, Ameae M. Walker. Division of Biomedical Sciences, University of California, Riverside, CA, 92521 —0121, 951-827-5942 (Tel), 951-827-5504 (Fax), ameae.waiker@ucr.edu

ABSTRACT

Ovarian cancer cell lines have been reported to express prolactin (PRL) receptors, and PRL has been shown by others to be anti-apoptotic in ovarian cancer cells. However, multiple PRL receptors derived from differential splicing of the same gene transcript transduce very different PRL-initiated effects on cells. In this project, we determined the kinds of PRL receptor expressed by TOV-112D, OV-90 and TOV-21G cells. All three lines are epithelial and derived from malignant grade 3, stage III adenocarcinomas. Using quantitative real time PCR and Western blot, we established that the long form (LF) and both short forms (SF1a and SF1b) of the human PRL receptor were expressed by all cell lines and that the level of expression of a particular receptor type was comparable among cell lines. In order to clarify the functional role of these receptors, we used three different ligands for the receptor, one which in other systems promotes proliferative and anti-apoptotic responses (unmodified PRL), one which acts as a competitive antagonist since it binds to but does not activate the receptor (G129R PRL), and one which is a selective activator, inhibiting growth responses to unmodified PRL and yet promoting both apoptotic and differentiative responses (S179D PRL). When viable cell number was assessed at the end of a 3 day incubation in each ligand (500 ng/ml) in medium containing 10% FBS, unmodified PRL had no effect on cell number while both of the other ligands reduced cell number (15-35%). When FBS was omitted from the incubation medium and viable cell number was assessed after 6 days, unmodified PRL increased cell number (by 20-40%) while G129R PRL and S179D PRL decreased cell number by 10-15%. Further analysis of the TOV-112D cells showed decreased apoptosis in the 6 day assay with unmodified PRL (by 28%) and increased apoptosis in response to S179D PRL (by 10%). Evaluation of cell migration in a Transwell system showed unmodified PRL significantly promoted migration: The relative number of migrating cells was increased by 30% after 20h, while S179D PRL and G129R PRL decreased migration by 27% and 20%, respectively. When the cells were transiently transfected with a plasmid expressing the LF receptor, the increased survival response and increased migration with unmodified PRL were exaggerated; when transfected with a plasmid expressing the SF1b receptor, the decrease in cell number and decreased migration in response to S179D PRL was exaggerated. These results, while modest in the short term, suggest different roles for the LF and SF1b receptor, an important role for unmodified prolactin in survival and migration of ovarian cancer cells, and potential therapeutic applications for S179D PRL and G129R PRL. This work was supported by OCRP grant OC073294.